

# Primary and Secondary Risk Factors for Birth Defects

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Birth defects may be inherited in the germ line or may result primarily from a wide spectrum of predictable physical, chemical, and infectious processes that can operate in the mother, the father, or in the zygote. The systematic consideration of these mechanisms can lead to a fresh awareness of risk and possible strategies toward recognizing and avoiding such risks. Birth defects also depend heavily on secondary factors that may even be of greater concern than any single primary insult because they may simultaneously affect the consequences of more than one primary exposure. Under the influence of secondary factors, the frequency, timing, and intensity of developmental deficiencies can be quite varied. It is particularly interesting that expression can be delayed until quite late in life, and deficiencies may occur or be expressed only in response to the appropriate environmental stress or functional demand. Any discussion of teratogenic mechanisms, therefore, is not complete without taking into account the important concept of co-teratogenesis, or the operation of secondary risk mechanisms. The principle of secondary risk or co-teratogenesis has been demonstrated by means of enhancement of radiation-induced terata by the administration of drugs that inhibit DNA repair. An example of late-onset expression of prenatal damage was illustrated with postnatal retinal degeneration occurring after prenatal damage to the developing retina. It is suggested that a systematic consideration of primary and secondary risk mechanisms can lead to a better understanding of the problem of birth defects.

## Introduction

The purpose of this paper is to provide an overview of birth defects and their relationship to environmental insults. Such a discussion, if joined widely by environmental and biomedical scientists, can result in more coordinated and effective approaches to the prevention of environmentally provoked abnormalities through better understanding of the nature and management of risk resulting from exposure to either natural or man-made factors. Birth defects, of course, represent a restricted subset of significant toxic events targeting the conceptus that do not result in immediate death but still permit some progression of the developmental processes. In fact, failure of survival of the reproductive product must also be considered as a possible result of the same processes as those discussed here. This discussion emphasizes two major points: First, birth defects result from a wide spectrum of predictable physical, chemical, and infectious processes that can operate in the mother, the father, or the zygote. The systematic consideration of these mechanisms can lead to a fresh awareness of risk and possible strategies toward recognizing and avoiding such risks.

Second, the causes and expression schedules of birth defects depend heavily on secondary factors as well as primary mechanisms of damage. In fact, secondary fac-

tors may even be of greater concern than any single primary insult because they may simultaneously affect the consequences of more than one primary exposure. The discussion of secondary factors should be prefaced by four generalizations: *a)* The outcomes for perturbations of the developmental processes are dictated by the basic critical vulnerabilities of the targeted systems; *b)* the timing and intensity of developmental deficiencies can be quite varied (immediate, delayed, subtle, mild, severe, or latent), with resulting great increases in the time of vulnerabilities for compromised structure and function; *c)* expression of developmental deficiency may occur only in response to the appropriate environmental stress or functional demand; and *d)* any discussion of teratogenic mechanisms, therefore, is not complete without taking into account the important concept of co-teratogenesis, or the operation of secondary risk mechanisms. The following is a general review of these principles and some brief illustrations of some of the concepts from our own work.

## Classification of Birth Defects in Relation to Prevention Strategies

"Birth defects" may be defined as abnormalities of biological form and/or function resulting from abnormal/incomplete differentiation and development. "Teratogenesis" generally is used to refer to the processes involved in development of such abnormalities. Birth defects can be

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classified in a number of different ways, and the use of different schemes has led to some general confusion about causes and mechanisms. The most important consequence of any approach to the general subject is clarity in our thinking about how we can prevent or modify the severity of such phenomena.

## Inherited Birth Defects

Defects that are simply passed along in the germ line from either parent are classified as *inherited defects* or deficiencies. These defects, according to the principles stated above, can be large anatomical defects or simple enzyme/metabolic defects; they may be expressed immediately at birth or may be delayed in expression until much later in life; or they may not even be detected until or unless the individual is stressed appropriately. Furthermore, the extent or nature of their expression may depend on the additional genetic makeup of the individual. In many instances defects may even be considered as "normal variants."

It is particularly interesting to consider those defects and diseases that are not detected at birth and whose consequences are delayed and appear progressively in postnatal life. These diseases are not uniform in the age of onset nor in the severity of the resulting disease manifestations, suggesting a potential role of environmental factors. Mechanistically, expression depends on time and the environment in specific ways, and better understanding of these facts can lead to control strategies. The hope of modern gene technology is that some deficiencies can be corrected directly by supplying the genes for the missing proteins. Even without success with such innovative techniques, it may still be possible to modify the outcome by other specific rational approaches. When an essential synthetic enzyme is deficient, it may be possible to provide the essential product through direct supplementation or to activate or stimulate alternative metabolic pathways. In the case of defects in catabolic enzymes or enzymes that eliminate toxic metabolic intermediates, the time of disease development depends on the kinetics of the system because there must be sufficient time to allow accumulation of the corresponding metabolic product(s). Control could take the form of decreasing the synthesis of the precursor of the product through targeted metabolic control, preventing exposure to precursors from the environment, or by activating alternative degradative pathways.

When degenerative changes are involved, time and molecular turnover must occur to generate the deficiencies that account for losses in function. In this instance the therapeutic or preventive objectives could be met by decreasing turnover through stabilizing the target molecules or by stimulating synthetic and reparative processes. Furthermore, abnormal synthetic processes may not be activated and become significant until the appropriate level of maturity is achieved that requires the expression of the corresponding synthetic process. This may allow for therapeutic interference with the activation of the targeted process. Degeneration or toxic manifestations may not occur until the appropriate stress or demand condi-

tions are presented to trigger evidence of defective function, providing the opportunity to intervene through understanding and controlling such stress. It is important, therefore, to recognize that there are environmental and temporal constraints and influences on the expression of inherited birth defects. Through recognition of the full spectrum of primary and secondary risk factors, both intrinsic and environmental, it should be possible to modify the occurrence and expression of many such defects. The main point to be made is that even the classic inherited type of birth defect or genetic disease can be sensitive to environmental manipulation and should be of great interest and concern to the environmental scientist.

## Acquired Birth Defects

The birth defects that have attracted the most attention of environmental scientists are those that may be considered as acquired or inflicted through the action of physical, chemical, or infectious agents in the environment. These may be further subdivided into three groups.

First, abnormalities may result from events that compromise the information provided by either of the parental germ cells at some point before fertilization. This recognition that the differentiation process is vulnerable to events that impinge on either parent is essential to understanding environmental risk and contrasts with the historical tendency to consider as targets only the developing zygote and/or the egg. Thus, either germ cell may acquire a new mutation, there may simply be maldistribution of genes and organelles during the processes of cell division (both reductive and nonreductive), or maturation can be disrupted. It is interesting to contrast the possible differences in targeting male and female germ cells in mammals, in view of the fact that stem cells continue to yield progeny throughout reproductive life of the male, whereas in the female, differentiation is essentially complete prenatally, and only final reductive divisions and maturation of the egg occur in the adult mammalian female. This means that male and female germ cells may differ greatly in their susceptibility to damage and their ability to repair and recover. Moreover, the long-term consequences from mutations of gamete stem cells in mature males contrasts with those from mutations in the differentiated gamete in the mature female because, in the stem cell, a mutation may influence multiple reproductive events rather than a single mature gamete.

There is a general tendency in thinking about mutational causes of birth defects to look for specific syndromes or complexes of abnormalities. This is taking the most specific and restricted view of differentiation: that abnormalities of a single system can only arise from a few mutations in a limited number of genes, such as those that directly control the sequence of development. However, when development is considered as a balanced assembly of processes, functions, and cell interactions, it is easy to see that even in relation to differentiation and function of a specific organ system a large variety of mutations could be expected to have an influence, and each, if it survived, could result in a different slate of defects. It may be

fallacious, therefore, to look for specific developmental syndromes associated with specific mutagenic actions and agents.

The female gamete has an additional vulnerability over that of the male because of the fact that the mitochondrial genome of the zygote is contributed entirely by the egg. This is complicated further by the fact that mitochondria are not equipped with the same DNA repair mechanisms as the nucleus. It is not possible to assign the relative frequency of teratogenic events that arise from compromise of the germ cells, but awareness of their vulnerability is essential to any prevention strategies.

Second, any significant compromise of the zygote can result in developmental disruption. Insults may be in the form of damage to DNA, maldistribution of genes and organelles during fertilization and subsequent cell division, critical changes in cell numbers arising out of modification in cell division and turnover, or interference with membrane transport, metabolism, metabolic control, and other normal regulatory processes within the zygote (cell-cell communication, and autocrine, endocrine, and neural control generally). Furthermore, the zygote is vulnerable to physical perturbations such as mechanical pressures during differentiation, as well as a large number of unknown infections. When changes in the DNA are involved, these may be termed, in the broadest context, somatic cell mutation. However, it must be understood that it does not follow that persistent genetic changes will be detectable in the surviving cells of the zygote, indeed, even in abnormal organs. The mutated cell(s) simply may not survive, and the zygote may be compromised in its ability to develop fully and correctly.

Third, highly differentiated animals depend on very specific functioning and relationships with the support structures in the placenta (or the yolk sac in nonplacental animals). Thus, any disruption with the orderly and appropriate exchange of gases, nutrients, and waste materials either on the maternal side or at the zygote can lead to significant fetotoxicity.

Attention must be focused, therefore, on the germ cells, the zygote, and the placenta in a complete discussion of the causes and management of acquired birth defects. These generalizations are summarized in Table 1.

**Table 1. Types of birth defects  
(anatomical or biochemical/functional deficiencies).**

|  |
|--|
| Inherited  |
| Expression immediate, delayed, or latent   |
| Expression may depend on additional genetic makeup   |
| Expression may depend on stress or other environmental factors                               |
| Timing and contingency of expression provides opportunity for intervention                   |
| Acquired   |
| Insults to germ line cells   |
| Mutations and maldistribution of genes and organelles  |
| Interference with maturation   |
| Insults to zygote  |
| Mutations and maldistribution of genes and organelles during and subsequent to fertilization |
| Influences on cell numbers and survival  |
| Regulation of cell and tissue processes  |
| Interference with placental function   |

## Targets for Teratogenic Insults Leading to Acquired Birth Defects

The general targets for teratogenic insults are summarized in Table 2. Undoubtedly, DNA is the most effective target of all. It is the largest unique molecule in the cell, and has extremely low copy numbers for most of its functions. It is both accessible and chemically reactive. It has hydrophobic regions to bind nonpolar ligands, is a highly ordered polyelectrolyte with extensive opportunity to react with charged species, and it presents, for reaction, electrophiles, nucleophiles, and aromatic rings. Because of its central information role, rare lesions can be amplified into large biological events, and lesions can be stored for later expression. DNA damage is the most predictable embryotoxic mechanism. It would be expected, therefore, that agents known to be mutagens or to interact significantly with DNA to modify expression should be very suspect as potential teratogens. It is perhaps fortunate that significant damage to the genome probably results in a nonviable zygote.

DNA is a complicated target for a number of reasons. For a specific structural gene, the consequences of alteration are straightforward, ranging from complete abrogation of function to changes in efficiency, specificity, regulation, or other characteristics of the resulting biological roles. For a regulatory gene, alteration may result in loss of normal regulatory control, an event of great significance to the process of differentiation. In addition, vast sequences of DNA are noncoding in the usual sense, and the results of small changes in structure are unknown. Furthermore, there exist in most cells highly active repair systems whose function is to restore the structural integrity of DNA. Although these would seem to be of great importance in preserving the differentiation process, their precise roles in either the germ cells or the zygote are not known. Despite these complexities, DNA may be presumed to be an important target in assigning risks during differentiation. Presumably, the consequences of damage depend, among other things, on the developmental stages of the targeted cells and the recovery potential through molecular repair, simple cell replacement, and tissue repair and remodeling. The roles for expression of viral genomes or their interference with normal expression must also be considered in any discussion of such nucleic acid targets.

In addition to the information system, the requirements for structural and functional integrity suggest several additional classes of targets. Clearly, any disruption of cell organelles and their structural molecules would be critical to both germ cells and the zygote. Similarly, cytoskeletal

**Table 2. Targets for teratogenic insults.**

|   |
|---|
| DNA: single most effective target         |
| Organelles and their structural molecules |
| Cytoskeletal proteins                     |
| Critical catalytic proteins               |
| Regulatory molecules                      |
| Extracellular structural macromolecules   |
| Membranes and transport proteins          |

proteins are critical to the structural integrity of both cells and tissues. Critical catalytic proteins (enzymes, contractile and transport proteins) are also essential. In the case of the zygote, both extracellular structural proteins and regulatory molecules play vital roles in differentiation and in establishing and maintaining structure. Thus, it is apparent that a series of potential targets may readily be identified from which damaging interactions may be translated into the development of birth defects.

## Targets for the Sites and Mechanisms of Action of Secondary Risk Factors

Understanding the fact and the mechanisms for operation of secondary risk factors in the genesis of birth defects is possibly of even greater importance than the recognition of single primary risk mechanisms. First of all, the action of any primary insult can be influenced drastically by the action of other factors, up to and including the possibility that a specific primary insult may result in birth defects only when coupled with the appropriate secondary conditions. Second, a secondary risk factor may simultaneously influence the consequences of exposure to multiple primary insults. A secondary mechanism, therefore, may amplify the effects either of multiple, primary environmental exposures or intrinsic deficiencies which if encountered alone might not have significant consequences. Finally, it should be emphasized that almost all attempts to recognize risk simply look for evidence of reproductive and developmental toxicity without considering the effects of any secondary risk mechanisms or possible effects of new agents on the ability of other preexisting primary risk factors to cause birth defects. Although our consideration of secondary risk factors or co-teratogenic mechanisms is very primitive, it is quite reasonable to think of the process in terms of possible targets and putative mechanisms. Table 3 provides a list of potential "co-teratogenic" mechanisms for discussion. In addition to these classes of environmental effects, it must also be remembered that host susceptibility to both primary and secondary risk factors may vary considerably due to

genetically determined differences in the targeted individuals.

The first category of co-teratogenic mechanism includes all those that exert their effects on the primary agent itself and its bioavailability in active form for presentation to relevant targets. In the temporal sense, these are the factors that operate before the teratogenic insults. These include such processes as metabolic or chemical activation or deactivation in the environment; influences on exposure, absorption, excretion, transport, and thus bioavailability in the target subject; and metabolic activation and inactivation in the exposed subject.

All these effects can be considered dose-modifying effects. There are, in fact, quite interesting and predictable consequences for such effects. In the case of chemicals released into the environment, it may be of major importance to consider the consequences of transformation either to inactive substances or from biologically inert into biological hazardous substances. Figure 1 illustrates a rather graphic example of the sort of concern that should be faced when releasing material into the environment. Thalidomide, as shown, is a phthalamide derivative that is a potent human teratogen. Also shown are examples of phthalamide pesticides that have been released into the environment in millions of tons. Fortunately, no biological or chemical transformation has occurred in the environment to result in an active phthalamide teratogen. However, this is the type of situation in which complete consid-

Table 3. Coteratogenic mechanisms.

|  |
|--|
| Mechanisms directed at offending agent ("dose" of ultimate insult)                 |
| Environmental processing and availability  |
| Absorption   |
| Excretion  |
| Binding  |
| Metabolism   |
| Mechanisms involving target for the insult   |
| Accessibility  |
| Number   |
| Expression   |
| Mechanisms affecting on response patterns  |
| Kinetics, demand, and accessibility of primary and interrelated functional systems |
| Effects on recovery  |
| Molecular repair   |
| Cell replacement, proliferation, and tissue recovery                               |

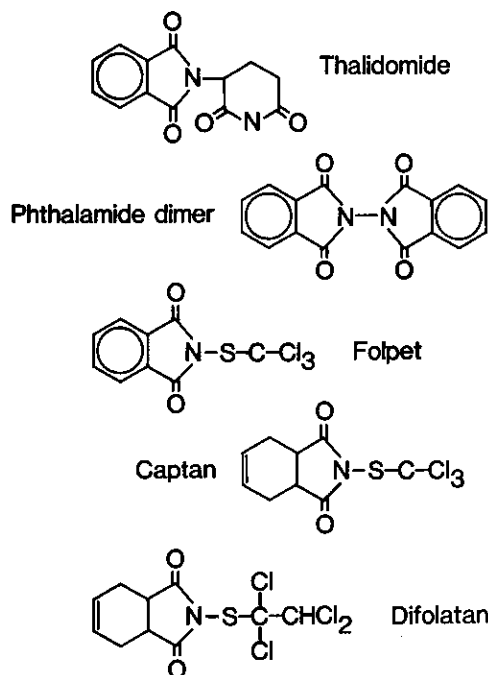


FIGURE 1. Comparison of the structures of thalidomide, a teratogenic dimeric derivative of thalidomide, and certain phthalamide-derived pesticides.

eration of risk mechanisms could suggest appropriate tests before release.

The evidence is much more compelling that chemical compounds can significantly influence the bioavailability of active forms of other agents through effects on activating or inactivating drug-metabolizing enzymes or drug-conjugating enzymes in the targeted host. Such effects from dietary intake, environmental exposure, or drug administration have been discussed extensively in relation to carcinogenic chemicals and drugs in general but have not been emphasized extensively for their relevance to teratogenesis. Such effects are excellent examples of how a single secondary agent can influence the biological effects of exposure to a variety of other agents. Alternatively, it is easy to see how such an effect on drug-metabolizing enzymes could result in conversion of insignificant effects of background chemicals into new biological consequences such as detectable levels of teratogenesis.

Secondary effects on targets and their function may also be important. For example, where specific receptors are involved, any secondary process that regulates receptor number and availability could influence the effectiveness of a primary insult. Enhanced risk may also result from simultaneous exposure to two agents that affect parallel targets representing processes that serve interrelated functions.

The last major category of co-teratogenic effects occurs after damage and includes all of the possible recovery processes. The first type of recovery is molecular repair such as in DNA after a variety of types of damage. Thus, lesions in DNA that are putatively lethal or mutagenic are simply converted back to normal DNA sequences through a series of enzymic steps. Just as a single DNA lesion can be amplified enormously during biological expression, prevention of repair of a single DNA lesion by exposure to some secondary risk factor can have an enormous influence on teratogenic consequences of DNA damage. This is particularly interesting in light of the background rates of DNA damage that occur as a consequence of metabolic by-products, irradiation, natural chemicals, etc. Thus, a powerful inhibitor of DNA repair could have the apparent effect of causing birth defects or of enhancing the effects of other gene-damaging agents. Other types of molecular repair, such as membrane assembly and reassembly, may also have biological reality at some level, and could be targets for secondary effects. Recovery from injury also includes the processes of cell turnover and replacement, which can also be vulnerable to secondary risk factors.

The conclusion to be drawn from these discussions is that any consideration of environmental risk must take into account the effects of a variety of secondary risk mechanisms. In the case of environmental teratogenesis, the concept of co-teratogenesis must be given at least equal attention, while for inherited birth defects, expression may be determined by environmental factors operating through some of these same mechanisms. The excitement of these insights is that strategies may be possible that can lead to prevention of expression of both acquired and inherited defects through appropriate manipulation of secondary factors.

## Modeling Environmental Co-Teratogenesis: Effects of Putative DNA Repair Inhibitors

Some years ago, our laboratory explored the concept of co-teratogenesis through the use of prenatal ionizing irradiation in conjunction with putative DNA repair inhibitors (1). These experiments were based on the observations that such irradiation and the resulting DNA damage predictably led to an array of birth defects whose form and severity were very sensitive to dose and the gestation time at treatment. We had also observed that the amino-quinoline antimalarial, chloroquine, and caffeine could inhibit DNA repair *in vitro* and could enhance X-ray lethality in mice. We reasoned, therefore, that inhibitors of repair might be used to enhance the teratogenic effects of irradiation or other types of DNA damage.

The experiments performed used standard methods of timed pregnancies and radiation exposures and assayed for cleft palates. Three findings were documented. First, chloroquine and caffeine alone were not teratogenic over the specific time schedule of these experiments, even at doses that were severely toxic to the mother. Second, chloroquine and caffeine administered after X-irradiation enhanced by 4-fold the number of cleft palates produced (1) over those from X-rays alone. Parallel experiments performed with neutron irradiation, which, in contrast to X-irradiation, produces nonrepairable double-stranded DNA strand breaks, resulted in no enhancement by concomitant chloroquine and caffeine administration (unpublished observations). Third, additional experiments using cytoxan as the teratogenic insult also showed similar enhancing effects of the chloroquine administration.

Although it can only be suggested circumstantially that the effect of the chloroquine was through inhibition of DNA repair, it may be concluded that enhancement of the teratogenic effects of irradiation and alkylation damage did occur in this mouse test system by some mechanism. Chloroquine and caffeine, therefore, acted as co-teratogens with irradiation. A question to be addressed is the role of the regulation of DNA repair mechanisms in determining possible effects on differentiation of the DNA damage that is occurring continually under ambient conditions even without the imposition of an acute external perturbation. While an additional slight change in the extent of DNA damage resulting from an acute, externally applied insult might not be significant, a change in the ability of the organism to repair intrinsic damage might result in the production of abnormalities. In such a scenario, the co-teratogenic mechanism would be the driver of the biological consequences. A systematic consideration of such mechanisms could be very important.

## Dose Effects of a Direct-Acting Alkylating Agent Administered Late in Pregnancy: Discovery of a Late-Onset Model of a Birth Defect

For additional studies of teratogenesis through DNA damage, it was desirable to develop a model with a defin-

able chemical lesion on the DNA inserted on precise time schedule and involving tissues sufficiently accessible for biochemical experiments. For these reasons, we undertook studies of the teratogenic effects of methylnitrosourea (MNU), a direct-acting alkylating agent. This reagent does not require metabolic activation, in contrast to cytoxan, and has a half-life of only a few minutes in biological fluids. Furthermore, it has been shown to interfere substantially with the differentiation of the retina, which occurs in the mouse beyond the day 16 of gestation. Thus, mouse embryos may be treated with a single pulse of damage administered to the mother, and the embryo is sufficiently developed to provide access to target organs for isolation and detailed study. It is also relatively easy to evaluate morphologic disruption in the retina because of its highly ordered structure.

A single high dose of MNU (20 mg/kg) on the day 16 of pregnancy in the mouse characteristically results in an abnormal retina with severe rosette formation and dysplasia. In a quest for a dose level that might cause rosettes only if accompanied by inhibition of DNA repair, we performed a series of dose titrations. Dose reduction below 5 mg/kg was required to achieve normal development of the retina. The exciting consequences of this series of experiments, however, was the observation that while the retinas appeared normal shortly after birth following MNU at 1 mg/kg on day 16 of pregnancy, many of these animals exhibited progressive loss of retinal layers throughout the first year of life (2). Thus, MNU at low dose produced a late-onset retinal degeneration. It is interesting that most studies of teratogenic potential simply assess animals at one time point, usually near the end of gestation. What is most interesting about this lesion is that the long-term stability of this nondividing tissue had been compromised by prenatal damage. Based on this sort of model, it is possible to expand considerably our concept of the consequences of prenatal defects. Thus, in either inherited or acquired birth defects the possibility must be considered that abnormalities may be expressed only at times later in life, possible in the form of a degenerative process. When complicated evaluation of behavioral consequences of developmental damage are included within the framework of this discussion, the implications are especially far reaching.

The acquired defect with low-dose MNU, like certain inherited defects of the retina and other organs, appeared as a long-term instability and degeneration. Accordingly, it would seem that this process might also be sensitive to environmental factors. In fact, the retina in the mouse, as well as in other species, is quite sensitive to the effects of light irradiation due to damage by singlet oxygen. Accordingly, our experiments showed that the MNU effect at low but not at high dose was very sensitive to light conditions. We showed that rearing animals in total darkness completely prevented the degeneration, while constant

illumination caused rapid atrophy of the retinas in the control animals as well (3). Furthermore, it was found that maintenance of the animals on high levels of the antioxidant butylhydroxytoluene could prevent the combined effects of MNU and moderate illumination (4), presumably by scavenging singlet oxygen.

These experiments established two important points. First, prenatal damage can produce biochemical lesions, which even in terminally differentiated cells can result in instability manifest by premature or accelerated degenerative changes late in the postnatal period. Second, and even more exciting, is the conclusion that through an understanding of the expression factors, it may be possible to prevent the development of overt disease even in the face of defects developed prenatally either from inherited defects or from environmental damage.

## Summary and Conclusions

I urge that we, as medical and environmental scientists, undertake a systematic consideration of primary and secondary risk mechanisms for the production of environmentally related diseases. In the case of birth defects, this should be framed as a study of both teratogenic and co-teratogenic mechanisms that contribute to the production and management of birth defects. I urge that the complete spectrum of birth defects to be considered include late-onset problems such as degeneration and disease susceptibility because defects cannot always be recognized immediately at birth either as gross anatomical or biochemical abnormalities. It is especially important to understand delayed expression because of the problem of relating cause and effect and because there may be an extended time during which there are opportunities to intervene and prevent overt disease. This can be important for both inherited and acquired defects. The experimental work reviewed is intended to serve only as illustrative examples. For details, the reader is referred to the original publications.

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